IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Attorney Docket:- NRDC-17

ANSON, D.S., et al Group Art Unit: 127

Serial Number: 06/839,215 Examiner:

Filed: March 13, 1986

For: FACTOR IX PROTEIN

Date: May 14, 1986

SUBMISSION OF PRIORITY CLAIM AND PRIORITY DOCUMENT IN ACCORDANCE WITH THE REQUIREMENTS OF RULE 55

Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

Sir:

It is respectfully requested that this application be given the benefit of the foreign filing date under the provisions of 35 U.S.C. 119 of the following, a certified copy of which is submitted herewith:

Application Number	Country of Origin	Filed
8506767	United Kingdom	March 15, 1985
8515416	United Kingdom	June 18, 1985

Respectfully submitted,

MURRAY AND WHISENHUNT

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I, the undersigned, being an officer duly authorised in accordance with Section 62(3) of the Patents and Designs Act 1907, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

I also certify that by virtue of a direction under Section 30 of the Patents Act 1977, the application is now proceeding in the name substituted as identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or the inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

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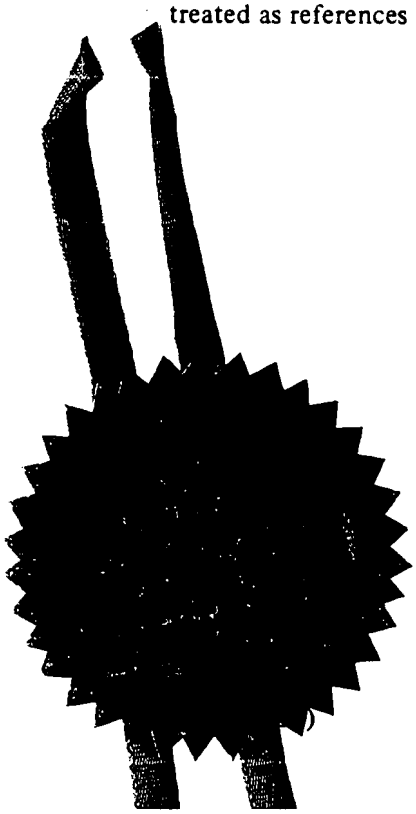

Please turn over

FACTOR IX PROTEIN

This is further to our UK patent application No.8506767 filed 15th March 1985, the entire contents of which are herein incorporated by reference.

The recombinant plasmid pIJ5/9, referred to in stage 3 of Example 2, in E.coli MC1061 has been deposited as a patent deposit under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the purposes of Patent Procedure, at the National Collection of Industrial Bacteria, Torry Research Station, P.O. Box 31, 135 Abbey Road, Aberdeen, Scotland AB9 8DG, on 13th June 1985 under the number NCIB 12103.

The invention includes specifically this expression plasmid pIJ5/9 and like plasmids constructed according to the general scheme of Figure 6 of Application 8506767, having similar DNA expression sequences and having a factor IX DNA sequence which codes for the same amino acid sequence (taking into account the degeneracy of the genetic code.)

The invention further includes specifically mammalian cells, especially from the preferred commercially available dog kidney cell line, transfected with a bacterial host, especially a transformation - competent E.coli, containing a plasmid pIJ5/9 or the like as defined above.

The invention also includes, of course, human factor IX protein produced from mammalian cells as defined above.

REQUEST FOR THE GRANT OF A PATENT (continuation sheet)

I AGENT'S REFERENCE 128728

III APPLICANTS

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AF2 ATTACH

C.S.1

No. 8515416

By virtue of a direction given under Section 30 of the Patents Act, 1977, the application is proceeding in the name of

National Research Development Corporation.
101, NEWINGTON CAUSEWAY
LONDON
SE1 6BU.

VII Declarati

VIII The Appl

Earlier application or patent number ..... and filing date .....

18 JUNE 1985

PATENTS ACT 1977

PATENTS FORM No. 1/77 (Revised 1982)

(Rules 16, 19)

18/06/85 A1198 PAT*** 10.00

1985
15416

The Comptroller
The Patent Office
25 Southampton Buildings
London, WC2A 1AY

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REQUEST FOR GRANT OF A PATENT

8515416

HE GRANT OF A PATENT IS REQUESTED BY THE UNDERSIGNED ON THE BASIS OF THE PRESENT PPLICATION

Applicant's or Agent's Reference *(Please insert if available)* 128728

Title of Invention FACTOR IX PROTEIN

Applicant or Applicants *(See note 2)*

Name (First or only applicant) DONALD STEWART ANSON

APPLICATION FILED 29.1.86

Country ..... State ..... ADP Code No. .....

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SECTION 30 (1)

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Continued on attached sheet

ventor (see note 3)

(a) The applicant(s) is/are the sole/joint inventor(s)
or
(b) A statement on Patents Form No. 7/77 is/will be furnished

ne of Agent (if any) *(See note 4)* Mr. R.K. Percy

ADP CODE NO

ress for Service (See note 5) Patent Department,
National Research Development Corporation,
101 Newington Causeway, London SE1 6BU

VII Declaration of Priority (See note 6)

Country	Filing date	File number

PATENT OFFICE
18 JUN 1985
RECEIVED BY

VIII The Application claims an earlier date under Section 8(3), 12(6), 15(4), or 37(4) (See note 7)

Earlier application or patent number ..... and filing date .....

IX Check List *(To be filled in by applicant or agent)*

A The application contains the following number of sheet(s)		B The application as filed is accompanied by:-	
1 Request	2 Sheet(s)	1 Priority document	
2 Description	1 Sheet(s)	2 Translation of priority document	
3 Claim(s)	0 Sheet(s)	3 Request for Search	
4 Drawing(s)	0 Sheet(s)	4 Statement of Inventorship and Right to Grant	
5 Abstract	0 Sheet(s)		

X It is suggested that Figure No ..... of the drawings (if any) should accompany the abstract when published.

XI Signature *(See note 8)*

R.K. PERCY, Chartered Patent Agent, Agent for the Applicants

NOTES:

1. This form, when completed, should be brought or sent to the Patent Office together with the prescribed fee and two copies of the description of the invention, and of any drawings.
2. Enter the name and address of each applicant. Names of individuals should be indicated in full and the surname or family name should be underlined. The names of all partners in a firm must be given in full. Bodies corporate should be designated by their corporate name and the country of incorporation and, where appropriate, the state of incorporation within that country should be entered where provided. Full corporate details, eg "a corporation organised and existing under the laws of the State of Delaware, United States of America," trading styles, eg "trading as xyz company", nationality, and former names, eg "formerly [known as] ABC Ltd." are not required and should not be given. Also enter applicant(s) ADP Code No. (if known).
3. Where the applicant or applicants is/are the sole inventor or the joint inventors, the declaration (a) to that effect at IV should be completed, and the alternative statement (b) deleted. If, however, this is not the case the declaration (a) should be struck out and a statement will then be required to be filed upon Patent Form No 7/77.
4. If the applicant has appointed an agent to act on his behalf, the agent's name and the address of his place of business should be indicated in the spaces available at V and VI. Also insert agent's ADP Code No. (if known) in the box provided.
5. An address for service in the United Kingdom to which all documents may be sent must be stated at VI. It is recommended that a telephone number be provided if an agent is not appointed.
6. The declaraton of priority at VII should state the date of the previous filing and the country in which it was made and indicate the file number, if available.
7. When an application is made by virtue of section 8(3), 12(6), 15(4) the appropriate section should be identified at VIII and the number of the earlier application or any patent granted thereon identified.
8. Attention is directed to rules 90 and 106 of the Patent Rules 1982.
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10. Applicants resident in the United Kingdom are also reminded that, under the provisions of section 23 applications may not be filed abroad without written permission or unless an application has been filed not less than six weeks previously in the United Kingdom for a patent for the same invention and no direction prohibiting publication or communication has been given or any such direction has been received.

C.S.1.

No. 85 06767

By virtue of a direction given under Section 30 of the Patents Act, 1977, the application is proceeding in the name of

National Research Development Corporation.

101, NEWINGTON CAUSEWAY
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PATENTS ACT 1977

15. MAR 1985

PATENTS FORM No. 1/77 (Revised 1982)

(Rules 16, 19)

The Comptroller
The Patent Office
25 Southampton Buildings
London, WC2A 1AY

1985
06767

18/03/85 A2514 PAT*** 10.00

REQUEST FOR GRANT OF A PATENT

8506767

THE GRANT OF A PATENT IS REQUESTED BY THE UNDERSIGNED ON THE BASIS OF THE PRESENT APPLICATION

I Applicant's or Agent's Reference *(Please insert if available)* 128180

II Title of Invention FACTOR IX PROTEIN

III Applicant or Applicants *(See note 2)*

Name (First or only applicant) DONALD STEWART ANSON

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V Name of Agent (if any) *(See note 4)* Mr. R.K. Percy | ADP CODE NO

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APPLICATION FILED 09.1.86

0444

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4 Drawing(s)	~~6~~ 5 Sheet(s)	4 Statement of Inventorship and Right to Grant	
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XI Signature *(See note 8)* Percy

R.K. Percy, Chartered Patent Agent, Agent for the Applicant(s)

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2. Enter the name and address of each applicant. Names of individuals should be indicated in full and the surname or family name should be underlined. The names of all partners in a firm must be given in full. Bodies corporate should be designated by their corporate name and the country of incorporation and, where appropriate, the state of incorporation within that country should be entered where provided. Full corporate details, eg "a corporation organised and existing under the laws of the State of Delaware, United States of America," trading styles, eg "trading as xyz company", nationality, and former names, eg "formerly [known as] ABC Ltd." are not required and should not be given. Also enter applicant(s) ADP Code No. (if known).
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(6849) Dd 8818957 50M 5/84 SP&D Gp 606

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FACTOR IX PROTEIN

Background of the invention

1. Field of the invention

This invention relates to factor IX protein, a protein involved in the blood-clotting mechanism of warm-blooded animals, and its production by recombinant DNA technology.

2. Description of prior art

Haemophilia B, or Christmas disease, is an inherited, X-linked bleeding disorder caused by a defect in clotting factor IX. Injection of factor IX concentrate obtained from blood donors allows most patients to be successfully managed. However, due to impurities in the factor IX concentrate in use at present, this treatment involves some risk of infection by blood-borne viruses such as non-A, non-B hepatitis virus and the virus that causes AIDS. Because of the recent concern over the increasing incidence of AIDS amongst haemophiliacs, a factor IX preparation derived from a source other than blood is desirable.

Factor IX DNA was cloned in 1982, see K.H. Choo et al., Nature 299, 178-180 (1982), K. Kurachi et al., Proc. Nat. Acad. Sci. USA 79, 6461-6464 (1982) and European Patent Application 107278 (NRDC). The work is summarised with sequence data and genome maps by D.S. Anson et al., EMBO J., 3 1053-1060 (1984) as well as in the above Patent Application.

Factor IX is a plasma glycoprotein which plays an essential role in the middle phase of the intrinsic clotting pathway where, in an activated form, IXa, it interacts with factor VIII(C), phospholipid and calcium ions to form a complex that converts factor X to Xa. Factor IX is synthesized in liver hepatocytes where the primary translation product undergoes three distinct types of post-translational modification before secretion into the blood-stream as a 415 amino acid long mature, biologically active protein.

These modifications are the vitamin K-dependent carboxylation of twelve glutamic acid residues, the addition of several carbohydrate residues and the beta-hydroxylation of a single aspartic acid

residue. The first two modifications are known to be required for activity. Because of the complex and specialized nature of these modifications, it seemed probable that the expression of active factor IX derived from factor IX DNA clones, would present great problems.

Summary of the invention

It has now been found possible to produce an artificial biologically active factor IX protein by recombinant DNA technology. The invention accordingly provides artificial factor IX protein, especially human factor IX protein.

According to an important aspect of the invention artificial factor IX protein is prepared by a process which comprises preparing a recombinant expression vector by linking a factor IX DNA sequence to a promotor sequence effective to express the DNA in a eukaryotic cell incorporating these DNA sequences in a vector, and introducing this expression vector into eukaryotic, preferably mammalian, cells in vitro, the eukaryotic cells having post-translational modifying means effective to modify the biologically inactive product of the expression of the DNA into biologically active factor IX protein.

The invention also provides a recombinant expression vector for use in the above process, comprising a factor IX DNA sequence linked to a promotor sequence effective to express the DNA in a eukaryotic cell, incorporated in a vector.

Brief description of the drawings

Figure 1 shows schematically a recombinant expression vector which can be used to transfect mammalian cells and which contains human factor IX DNA;

Figure 2 is a graph showing factor IX levels expressed from a monolayer of confluent mammalian cells transfected with the expression vector of Figure 1;

Figure 3 shows schematically the formation of another recombinant expression vector which can be used to transfect mammalian cells and which contains human factor IX DNA;

Figure 4 is a graph showing factor IX levels expressed from a monolayer of confluent mammalian cells transfected with the expression vector of Figure 3;

Figure 5 is a line diagram of a DNA fragment used in constructing the vector of Figure 1; and

Figure 6 shows schematically the method of construction of a vector pSVTKneo used as a starting material in the construction of an expression vector for use in the invention.

Description of the preferred embodiments

The invention is applicable primarily to human factor IX, although it could in principle be applied to animal factor IX. For example, bovine factor IX complementary DNA cloned sequences are described in the NRDC European Patent Application supra and they could be used to isolate full-length bovine complementary DNA clones, which could be expressed in a similar manner to that described here for human factor IX genes.

The term "factor IX DNA" is used herein to mean a DNA complementary to factor IX mRNA or derived from the exon regions of the factor IX gene by artificially removing intron sequences, which would yield upon translation in vivo a primary protein which can be modified by carboxylation of its glutamic acid residues, addition of carbohydrate residues and, if necessary, hydroxylation of an aspartic acid residue as mentioned above, to give a biologically active factor IX protein.

The factor IX DNA employed can be any likely to yield factor IX after modification. It is thought likely that it will normally include the cDNA complementary to that part of the mRNA which codes for the primary translation product. This includes the sequence coding for amino acids minus 1 to minus 46 designated as "PRE" and "PRO" by Anson et al., supra, Figure 6 on page 1058. "PRE" amino acids form a hydrophobic signal region (-46 to -21), "PRO" a hydrophilic precursor region (-20 to -1). "PRE" or "PRO" -coding mRNA sections could be replaced by foreign sequences.

Conveniently therefore the DNA includes a sequence which codes for the precursor region of the primary translation product lying to the 5'-end of that part of the mRNA which gives rise to the mature (biologically active) protein. In addition, the DNA can include part of the genomic factor IX DNA sequence extending further

in the 5'-direction, beyond the precursor-coding region. It will also be appreciated that the DNA can contain point mutations not affecting the amino acids coded for, as well as mutations, deletions and additions of nucleotides or short sequences thereof which alter the amino acids but do not materially affect the mature protein. As much as 10% of the DNA might be expected to be so varied.

For expression a eukaryotic cell or viral promotor is required. The Moloney Leukemia Virus LTR promotor and the SV40 early gene promotor have both been used successfully. The chosen promotor is linked to the 5'-end of the factor IX DNA. Ordinarily each will be present initially in its own vector. One of the vectors is restricted to excise the DNA and the other restricted to accommodate transfer thereto of the excised fragment, and the two are ligated. It would be expected that other viral promotors, such as the thymidine kinase gene promotor of Herpes simplex virus or the major late promotor of Adenovirus would be satisfactory. Equally the factor IX promotor could be used but might be less active than the viral promotors. An AAUAAA polyadenylation signal (N.J. Proudfoot and G.C. Brownlee, Nature 263, 211-214 (1976) is provided by the SV40 early gene polyadenlyation region of sequence, but others including the natural factor IX signal would be expected to function satisfactorily.

The vectors referred to herein are conveniently cloned into E. coli in manner known per se for bulking up.

The expression vector preferably also includes a selectable marker to enable selection of mammalian cells into which the factor IX gene has been introduced. The marker may comprise a prokaryotic DNA transposon sequence linked to a eukaryotic DNA promotor sequence, as for example in the TK/NEO gene.

In principle the kind of mammalian cell line most likely to be useful in the invention would be a hepatic cell or a transformed cell line derived from a hepatocyte. Unfortunately, none of the standard mammalian cell lines is known to secrete active endogenous factor IX. However, the rat hepatoma cell line H4-II-E-C3 (ATCC 1600) is known to secrete prothrombin which is gamma carboxylated

indicating the presence of a gamma carboxylase enzyme which might also serve to carboxylate the intermediate protein in the production of factor IX. This cell line has proved successful, but a commercially available dog kidney cell line not known to have any such modification "machinery" has proved to be even more successful. The cells must possess the necessary means of modifying the primary translation product or inactive protein to produce a protein which exhibits factor IX activity.

The introduction of the factor IX gene into mammalian cells may be carried out by the calcium phosphate precipitation technique. In this method a solution of the DNA to be transfected is made up in disodium phosphate. On the addition of calcium chloride, a fine precipitate of calcium phosphate is obtained in which DNA is trapped. After overlaying cells with this precipitate, some cells take up crystals of calcium phosphate along with the entrapped DNA. Within the cell the calcium phosphate crystals dissolve leaving the DNA free in some cases to integrate into the genome. Other known means of introducing DNA into cells are usable, such as protoplast fusion, W. Schaffner, Proc. Nat. Acad. Sci. 77, 2163-2167 (1980); electroporation, H. Potter et al., Proc. Nat. Acad. Sci. 81, 7161-7165 (1984) and infection with viruses, e.g. A.D. Miller et al., Proc. Nat. Acad. Sci. 80, 4709-4713 (1983).

The factor IX protein can readily be recovered from the medium in which the transfected cells are grown, without the need for lysis. The protein is preferably purified by affinity chromatography. For this purpose an antibody, preferably a monoclonal antibody to factor IX, is attached by conventional means to a support material on a column and the factor IX - containing product is adsorbed onto the column. To elute it an appropriate chaotrope or disrupting agent can be employed, and it has been found that a mixture of a high molar concentration of urea, say about 6-8M, with an inorganic salt, for example about 0.8 to 1.2M of an alkali metal chloride, in admixture therewith is particularly useful for this purpose.

The following Examples illustrate the invention.

EXAMPLE 1

The general scheme of the preparation and analysis of factor IX protein is as follows:-

1. Construction of a factor IX DNA sequence. A full length sequence of factor IX cDNA (complementary to factor IX mRNA) was prepared.

2. Construction of an expression vector designed to express factor IX protein in eukaryotic cells. This vector herein termed pMLV/NEO, was constructed from the following component elements.

(a) a promotor for expression of the long terminal repeat °(LTR) of the Moloney Leukemia Virus (MLV);

(b) the small t antigen intron of similar virus 40 vector (SV40);

(c) the early polyadenylation signal sequence from SV40; and

(d) the TK/NEO gene which provides a marker, by which those eukaryotic cells containing the desired foreign gene can be selected.

3. Insertion of the factor IX DNA sequence into the expression vector pMLV/NEO to give recombinant DNA plasmid pMLV IX/NEO.

4. Transfection of a mammalian cell line with the recombinant DNA plasmid pMLVIX/NEO, whereby the foreign DNA becomes integrated into the chromosomal DNA of the cells.

5. Confirmation of the secretion of factor IX by a one-stage clotting assay and by inhibition of the clotting activity by the addition of a monoclonal antibody to factor IX.

The preparation and analysis of the factor IX protein will now be described in more detail:

1. Construction of a factor IX DNA sequence

For a detailed understanding of this stage readers are recommended to refer to D.S. Anson et al., EMBO J. 3, 1053-1060 (1984). This paper contains extensive nucleotide sequence information on human factor IX DNA obtained from both factor IX MRNA ("cDNA") and from the human genome ("genomic DNA"). The genome is about 34 kilobases (Kb) long, which is more than 12 times the length of the mRNA, because the genome contains long intron sequences which do not code

for the RNA, interspersed with short exon sequences which do. The genomic organisation is shown in the Anson et al. paper.

The mRNA is 2802 residues long and contains a short, 29 nucleotide long 5' - non-coding sequence and an extensive, 1390 nucleotide long 3' -non-coding sequence.

The starting factor IX DNA is the cDNA clone cVI described in Figure 1 of the Anson et al. paper, containing DNA corresponding to mRNA nucleotides 25 to 1572, but with the first 15 nucleotides inverted so that they are read 3'-->5' instead of 5'-->3'. It was first necessary, therefore, to add nucleotides 1-25 and substitute nucleotides of the correct strand for the inverted ones.

The region from nucleotides 25 to 93 was removed from the factor IX cDNA cVI by digesting cVI (held in the plasmid vector pAT153/PvuII/8) with BamHI followed by "filling in" the overhanging ends with deoxynucleoside triphosphates using the Klenow fragment of DNA polymerase I (E. coli). After dephosphorylation with calf intestinal phosphatase, a partial digest with EcoRV was performed. EcoRV recognises the sequence GATATC at nucleotides 91-96. A partial digest was necessary because of the presence of an additional EcoRV site at nucleotide 508. The resulting linear fragment of about 4.9 Kb was purified by agarose gel electrophoresis.

A short (0.1 Kb) TaqI/EcoRV fragment was generated from the factor IX genomic DNA XI by digesting XI (also held in the plasmid vector pAT153/PvuII/8) with TaqI, "filling in" the overhanging ends with deoxynucleoside triphosphates as before, in the presence of tracer amounts of alpha-^{32}P dGTP and recutting with EcoRV. This labelled fragment, corresponding to nucleotides 288-386 of the genomic DNA, see Figure 4 of the Anson et al. paper, was separated from other TaqI and EcoRV fragments derived from the plasmid vector by 6% acrylamide gel electrophoresis.

The 4.9 Kb and 0.1 Kb long fragments were ligated together with T4 DNA ligase, the resultant recombinant cloned in E. coli was isolated and characterized by standard methods and designated p5'G/3'cVI. This reconstructed factor IX gene therefore contained the sequence corresponding to nucleotides 1-1572 of the mRNA

effectively adding nucleotides 1-25 to and correcting the rearrangement in the first 15 residues of clone cVI. It also contained 8 extra nucleotides, genomic DNA residues 288-295, derived from the presumed factor IX promotor region, but these residues alone are not sufficient to specify promotion. The factor IX DNA insert present in p5'G/3'cVI was removed by restriction with BamHI and HindIII and isolated by agarose gel electrophoresis.

2. Construction of expression vector pMLV/NEO

The construction started from the plasmid pTKMol TK1. This is a pAT153 plasmid containing the thymidine kinase (TK) gene as a marker and the Moloney Murine Leukemia Virus (MLV) Long Terminal Repeat (LTR) sequences. The plasmid was restricted with HindIII and BamHI to remove the TK coding section of sequence. The remainder of the molecule (pAT153 and MLV LTR) was purified by agarose gel electrophoresis. It was then used as a vector for cloning of an agarose gel-purified 0.9 Kb BamHI/HindIII fragment containing the SV40 small t intron and early gene polyadenylation signal which was derived from pSV1 (a gift from Dr Davey, Warwick). Figure 5 shows a line diagram of this approximately 0.9 Kb fragment which is composed of 3 parts. The first is a synthetic deoxyoligonucleotide sequence of 21 residues providing a TGA chain termination stop codon in each of the 3 possible reading frames. The second part derives from the MboI 610 long fragment from residues 4710-4100 of SV40 virus (see pp 799-841 in "DNA tumour viruses, Molecular Biology of Tumour Viruses", second edition, Part 2 (revised) Ed. J. Tooze, Cold Spring Harbor Laboratory, 1981). This fragment provides the small t antigen intron bounded by parts of the small t and large T antigen coding regions. The third part derives from the BclI site, originally at nucleotide 2770, but eliminated in the re-construction shown in site (residue 2553) of SV40 virus and contains the AAUAAA SV40 polyadenylation sequence at residue 2638 as well as parts of the large T antigen and VP1 coding region. The small t antigen intron was included in this expression vector in case this was required for expression of protein. However, the absence of this

element in the second expression vector (see Example 2, below) showed that it was unnecessary. Recombinants containing the 0.9 Kb fragment were identified by restriction mapping and one was chosen and designated pMLV. pMLV was restricted with PvuI and EcoRI, and the EcoRI ends blunt-ended by "filling in" using the Klenow fragment of DNA polymerase I. The 4.4 Kb PvuI/EcoRI fragment containing the MLV LTR, SV40 sequences and most of the pAT153 sequences was purified by agarose gel electrophoresis.

A thymidine kinase/neomycin-kanamycin (TK/NEO) gene was then prepared. Such a gene is described by F. Colbère - Garapin et al., J. Mol. Bio. 150, 1-4 (1981). It was constructed by linking the eukaryotic promotor region (EPR) for the transcription of the TK gene in Herpes Simplex Virus type I to the well known transposon Tn5 which codes for the enzyme aminoglycoside-3'-phosphotransferase type II (APH(3')-II) conferring Kanamycin resistance. When mammalian cell lines were transfected with this TK/NEO gene they exhibited resistance to the aminoglycoside G-418, an antibiotic, thus indicating that the APH(3')-II gene had become incorporated and the APH(3')-II enzyme expressed in the eukaryotic DNA of the mammalian cells. The TK/NEO gene is thus a valuable dominant selective marker in eukaryotic cells.

The TK/NEO gene and part of the amp-r gene was purified as a PvuI/BamHI fragment from the cosmid vector pTM, F.G. Grosveld et al., Nucleic Acids Research 10, 6715-6732 (1982). The BamHI end was made blunt-ended by "filling in" (see above). This fragment was then ligated to the pMLV-derived PvuI/EcoRI fragment using T4 DNA ligase and recombinants selected on ampicillin. Note that the PvuI site interrupts the amp-r gene so that in this ligation the amp-r gene was reconstructed in part from one fragment and in part from the other. The structure of recombinants was checked by restriction mapping and one clone, designated pMLV/NEO, was used in the next stage.

3. Insertion of factor IX DNA sequence into plasmid pMLV/NEO to form recombinant plasmid pMLVIX/NEO

The isolated factor IX DNA from Stage 1 was then ligated into the HindIII site of the expression vector pMLV/NEO from Stage 2 by

the use of T4 DNA ligase and the Klenow fragment of DNA polymerase I. Clones containing the factor IX DNA insert in the correct orientation were identified by restriction mapping, and one such clone, pMLVIX/NEO, was used in the next stage. Figure 1 of the drawings shows pMLVIX/NEO. The factor IX and TK/NEO transcription units are indicated by arrows which indicate the 5'---➔3' direction of the mRNA transcribed from these genes. (Note for lay readers: since the cDNA in the plasmid is double-stranded, it is meaningless to specify a direction of the DNA itself. Only one of the two strands would be transcribed into RNA and the arrow indicates the 3'---➔5' direction of the strand which is transcribed). pAT153 sequences are shown by the narrow filled line (1), the TK/NEO gene by the zig-zag line (2). The factor IX transcription unit, from 5' to 3', consists of the MLV LTR (solid triangles, 3) factor IX 5' non-coding sequence (open wide line, 4), factor IX coding sequence (filled wide line 5) part of the factor IX 3' non-coding sequence (open wide lines, 6) and SV40 sequences (diagonals, 7). The total length of the DNA is about 9.0 Kb.

4. Transfection of a mammalian cell line with the recombinant plasmid pMLVIX/NEO carrying a human factor IX gene

The rat hepatoma line, H4-II-E-C3 deposited at the American Type Culture Collection as an open deposit ATCC 1600 was transfected with the factor IX expression plasmid by calcium phosphate precipitation, as follows. The DNA of interest was made up in HBS buffer to a concentration between 10-50 micrograms ml^{-1}. The HBS buffer contains 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2PO_4, 5 mM glucose, and 20 mM Hepes pH 7.05. $CaCl_2$ was added from a concentrated (2.5 M) stock to a final concentration of 0.125 M. The mixture was left at room temperature for 30 minutes during which a fine precipitate formed. The precipitate was layered onto the washed monolayer of tissue culture cells and left at 37°C for 30 minutes. The monolayer was then overlayed with complete growth medium and incubation was continued overnight at 37°C. The following morning the medium and precipitate were removed and replaced with a solution of 15% glycerol.

After incubation at room temperature for 2-3 minutes the glycerol was removed and replaced with fresh medium. Cells containing a functional TK/NEO gene were selected for G418 resistance by the addition of G418 at 400 micrograms/ml 48 hours after the transfection and glycerol shock treatment and 24 of the resultant colonies were amplified by growing in 0.7 cm diameter wells of a 96-well multititre dish. Cells were removed from such wells by standard trypsin treatment, counted in a haemocytometer, diluted to about 3 cells/ml of medium and then 0.2 ml was dispensed into each well of a new 96-well dish. Colonies grew in about 10 weeks and one of each set was chosen as the permanent cell line. It was then grown up and the growth medium assayed for the presence of secreted human factor IX using an enzyme-linked immunosorbent assay (ELISA) as described in Stage 5 below. Four of the cell lines secreted detectable levels of factor IX. The highest producer, line 4A, was chosen for further analysis. Southern blot analysis indicated that there was only 1 copy of the plasmid integrated into chromosomal DNA per cell. Northern blot analysis of total cellular RNA derived from line 4A revealed the presence of a factor IX mRNA species of about 1.6 Kb, which is the expected size of the factor IX transcription unit. In both the Southern and Northern blot analysis no cross-hybridizing species was detectable in the control hepatoma cell line H4-II-E-C3.

5. Confirmation of the secretion of human factor IX

Cells were seeded at 10 confluency in a monolayer in an 80 cm^2 flask containing 10 ml of medium (MEM, 10% FCS containing penicillin, streptomycin, kanamycin and 100 ng/ml vitamin K). The medium was changed every 24 hours and the conditioned medium assayed by an ELISA for human factor IX. The ELISA was done by first binding 0.2 ml of a mouse anti-factor IX monoclonal antibody 3A6, diluted 1 in 5000 in 200mM sodium carbonate pH 9.0 to microtitre wells at room temperature for two hours. 150 microlitre samples of conditioned medium were then bound overnight at 4°C. The second antibody, 150 microlitres of rabbit anti-human factor IX (Calbiochem-Behring), diluted 1 in 500 in PBS, 1% NP40, 10% horse serum, was

then bound at room temperature for two hours. The third antibody, peroxidase-conjugated swine anti-rabbit IgG (Dako), was bound in the same manner. The assay was finally developed with 0.2 ml of 40mM ABTS (azinodi-(3-ethylbenzthiazole-sulphonic acid)-diammonium salt) in 0.008% H_2O_2 in 50mM citrate buffer pH 4.0 for 30 to 45 minutes. Colour change of the solution was then measured at 405nm in a spectrophotometer. A standard curve was constructed using 2-fold dilutions of normal plasma in the range of 10^{-3} to 10^{-5} dilution assuming a concentration of 5 micrograms/ml for plasma factor IX.

The secreted factor IX was tested for its adsorption onto barium sulphate by the procedure of K. Fujikawa et al., "Methods in Enzymology", Volume 45, Academic Press 1976, page 77. This procedure depends on the gamma carboxylation of the factor IX precursor and the positive values accordingly indicated gamma carboxylation.

Figure 2 of the drawings shows the time course of the secretion of human factor IX by cell line 4A together with the results of the barium sulphate adsorption test. The solid squares ■ indicate factor IX levels, the solid circles ● the levels of $BaSO_4$ - adsorbed factor IX and the open squares □ the degree of confluency of the cells. The rate of secretion of factor IX was about 6ng/10^7 cells/24hrs. As the recovery of factor IX is not corrected for possible losses in the procedure, it is not clear from this experiment alone whether about 70% of the factor IX has gamma-carboxyglutamic acid residues, or alternatively, that the material is all gamma-carboxylated and there are losses of about 30% in recovery. The fact that activity correlates well with antigen level (see below) indicates that the latter is the correct explanation). No factor IX was detectable in lysates of the 4A cells, indicating that there was insignificant accumulation inside the cell. No cross-reacting material was detected either in control H4-II-E-C3-conditioned media or in cell lysates.

Clotting and antigenic analyses of a sample of affinity-purified human factor IX from conditioned medium of line 4A cells as well as from a parallel control sample of H4-II-E-C3 cells were performed.

The human factor IX sample from 1.5 litres of conditioned media from line 4A cells, and the control sample from the same volume of H4-II-E-C3 cells were purified by barium citrate adsorption and immunoaffinity chromatography. An anti-human factor IX antibody affinity column was prepared by binding the 3A6 monoclonal antibody, purified from ascites fluid by sodium sulphate precipitation, to the support Affigel 10 (Biorad) using conditions recommended by the manufacturer. Thus the human factor IX sample to be bound was passed twice over the column (column volume = 0.3 ml) and it was then washed with over 100 volumes of PBS, 1% NP40 and a similar volume of PBS, 1M NaCl, 1% NP40. Finally the column was then rinsed with 5 volumes of PBS, 1M NaCl and eluted with a 7M urea, 1M NaCl eluant collecting 0.4 ml fractions. The first three fractions were pooled and immediately dialyzed overnight against 2% PBS at 4°C followed by freeze-drying. The samples were finally dissolved in 200 microlitres water. They were then assayed by ELISA as previously described and subjected to the clotting assay.

Factor IX clotting activity was measured, using the one stage clotting assay in which factor IX deficient plasma is used as a substrate see D.E.G. Austen et al., "A Laboratory Manual of Blood Coagulation", Blackwell Scientific Publications (1975). Human factor IX antigen was measured by ELISA. Both are given as percentages of average normal plasma levels. The inhibition effect of the anti-human factor IX monoclonal antibody 3A6 was measured by preincubation of the sample to be tested with a final concentration of 2.5% 3A6 ascites fluid for 15 minutes at room temperature.

Bovine factor IX antigen was measured in arbitrary units and was assayed by a quantitative ELISA as follows. Dilutions of pure bovine factor IX were diluted in 200mM sodium bicarbonate pH 9.0 buffer and dried onto microtitre plates. Bovine factor IX was then detected with a polyclonal rabbit anti-bovine factor IX antibody and a peroxidase conjugated swine anti-rabbit IgG antibody, as for the ELISA for human factor IX previously described.

Results of these analyses are shown in Table 1 below:

Table 1

Analysis of clotting factor IX activity and human and bovine factor IX antigen in purified samples from 4A and H4-II-E-C3 conditioned media

Assay	Cell Line 4A	H4-II-E-C3
Clotting (%)	7	1
Clotting (%) +3A6 monoclonal antibody	<1	<1
Human factor IX antigen (%)	6.8	not determined
Bovine factor IX antigen	0.8	1.2

The purified human factor IX was found to be active in a one stage clotting assay, giving an activity level of 7% of normal plasma. Only 1% activity was found in the control and this was presumably caused by the trace amounts of bovine factor IX which were found in both line 4A and control samples. An ELISA was therefore performed for bovine factor IX antigen and this indicated the presence of trace amounts in both line 4A and control cells. Further evidence that the cell line 4A is secreting active human factor IX was provided by determining the inhibitory effect on the human factor IX of the specific anti-human factor IX monoclonal antibody 3A6 in the clotting assay. Control experiments showed that this antibody does not significantly inhibit bovine or rat factor IX activity at the concentration used.

The fact that the activity level, 7% (of normal plasma), in the line 4A sample correlates well with the human factor IX antigen level, 6.8% (of normal plasma), suggests the protein is fully active. It is concluded from these results that line 4A cells secrete biologically active human factor IX.

EXAMPLE 2

In this Example a different mammalian cell line, one from a dog kidney, and a different promotor were used. A vast increase over Example 1 in the amount of factor IX produced was achieved.

Figure 3 of the drawings illustrates schematically the preparation of recombinant DNA containing the factor IX gene, as described in stages 1-3 below. As in Figure 1, arrows indicate the direction of transcription of the RNA.

1. Construction of a factor IX DNA sequence

This is as described in Example 1.

2. Construction of expression vector pKG5

pKG5 is a derivative of pSVTKneo, a plasmid expression vector which combines the SV40 early gene promotor and terminator derived from pSV2, see R.C. Mulligan et al., Science 209, 1422-1427 (1980), with the TK/NEO gene derived from the vector pTM, see Example 1.

The construction of pSVTKneo is illustrated in Figure 6. The plasmid pSV2 was digested with BamHI and PvuI, whereby two fragments were obtained. The smaller was discarded. The larger fragment (which contains a HindIII site) contained part of the ampicillin resistance gene, which was cleaved at the PvuI site, and the whole of the SV40 early gene promotor and polyadenylation sequence. The BamHI cleavage cut pSV2 at the end of the SV40 gene sequences. Separately, pTM was digested with the same enzymes and the larger fragment was discarded. The smaller fragment contained part of the ampicillin resistance gene, again cleaved at the PvuI site, and also the TKneo sequence which provides G-418 resistance (see Example 1, section 2). The BamHI site lies beyond the TKneo sequence. On ligation of the two fragments using T4 DNA ligase the amp-r gene is reconstructed and the TKneo sequence and SV40 sequence are assembled in the same plasmid.

The unique HindIII site in pSVTKneo was altered by the addition of an oligonucleotide to provide two further unique restriction sites for enzymes BamHI and XhoI, as described below. Plasmid pKG5 was constructed from the plasmid pSVTKneo. This construction was designed to add unique restriction sites to pSVTKneo in order to increase its general usefulness as an expression vector. Construction details were as follows: 1) pSVTKneo was digested to completion

with BamHI and blunt-ended using Klenow fragment of DNA polymerase I. The blunt-ended molecule was self-ligated and the resultant circular molecule transformed into E. coli. The resultant plasmid, designated pKG3, differs from pSVTKneo in that it has acquired four extra base pairs and, as a result has lost the BamHI site of pSVTKneo. 2) pKG3 was digested to completion with HindIII and blunt-ended with the Klenow fragment of DNA polymerase I. The resultant fragment was then ligated to a double stranded synthetic deoxyoligonucleotide:

(5')CTCGAGGATCCA(3')
(3')GAGCTCCTAGGT(5')

encoding the restriction sites for BamHI (GATCC) and XhoI (CTCGAG), and the ligation products were transformed into E. coli. From the transformants, a plasmid designated pKG5 was isolated with the unique restriction sites for HindIII (re-created upon successful ligation) BamHI and XhoI with the orientation of sites as shown in Figure 3.

3. Insertion of factor IX DNA sequence into plasmid pKG5 to form recombinant plasmid pIJ5/9

1 microgram of p5'G/3'cVI DNA was digested to completion by restriction enzymes BamHI and HindIII and the band containing factor IX sequences electroeluted from an agarose gel. About 100 ng of the eluted material was ligated with 100 ng of plasmid pKG5 that had previously been digested to completion by BamHI and HindIII. The ligation mixture was used to transform E. coli MC1061 and one transformant, pIJ5/9, was shown to have the desired genotype by detailed restriction analysis.

4. Transfection of a mammalian cell line with the recombinant plasmid pIJ5/9 carrying a human factor IX gene

pIJ5/9 was introduced into MDCK cells dog kidney cells available from Flow Laboratories Inc., by calcium phosphate - mediated transformation, see Example 1. pIJ5/9 contains the TK/NEO gene and selection was therefore made for G418 resistance. Three clones obtained from the transformation of one 9 cm diameter petri dish

were subsequently transferred into separate wells (volume of well = 2 ml) of a multi-well plate and allowed to grow to confluence. When confluence had been reached, the medium overlaying each clone was removed and analysed for the presence of human factor IX antigen.

5. Confirmation of the secretion of human factor IX

The media from the clones of dog kidney (MDCK) cells transformed with the recombinant DNA plasmid were assayed by sandwich ELISA (see Example 1). Two clones proved negative, whilst media from the third, labelled clone 593, contained human factor IX antigen at a level of more than 10 ng/ml (10 ng/ml was the upper limit assayable in this experiment). Furthermore, a sample of medium from clone 593 was adsorbed for 30 minutes in the presence of barium sulphate (see Example 1). After this incubation, the clarified medium was again assayed for factor IX antigen. 100% of the antigen present was removed by this pretreatment indicating the successful gamma-carboxylation of factor IX by MDCK cells.

In order to test further the factor IX product of clone 593, 50 ml of conditioned growth media were prepared as follows. Five 80 cm^2 flasks were each seeded with 593 cells in 10 ml of G418 medium. The flasks were left 4 days until they became confluent and the media were then harvested, pooled, and assayed quantitatively by ELISA for human factor IX antigen. The sample contained 60 ng per ml of factor IX, which is about 1.2% of the concentration found in normal human plasma.

50 ml of this material was purified by passing once through a factor IX monoclonal antibody immunoaffinity column (as described in Example 1) and the bound factor IX antigen was recovered in 1.5 ml of eluant. This preparation was assayed by ELISA and by clotting assay, as described in Example 1. The results of these assays are shown in Table 2, again as % of normal human plasma.

Table 2

Analysis of clotting factor IX activity and human factor IX antigen in a purified sample from clone 593

Assay	Amount
Clotting (%)	15
Clotting +3A6 antibody (%)	0
Human factor antigen (%)	15

The following conclusions can be drawn from the analyses:

(a) Human factor IX is produced and secreted by clone 593.

(b) After growth to confluence, 593 cells secrete at least 60 nanograms per ml factor IX antigen into the medium. This is gamma-carboxylated and retains its ability to bind the factor IX monoclonal antibody.

(c) Based on the binding to monoclonal antibody 3A6, 1.125 micrograms of human factor IX was recovered from a total of 3 micrograms applied to an immunoaffinity column (a recovery of 37.5%).

(d) The factor IX is fully active biologically as indicated by levels of antigen and clotting activity in the affinity-purified sample.

The amount of factor IX antigen produced by cell line 593 under normal growth condition was measured as follows. Cells of line 593 were seeded to a density of 50% confluence in a 25 cm^2 tissue culture flask. The culture was overlaid with 2 ml of growth medium and incubated at 37°C in a humidified incubator. At daily intervals the conditioned medium was removed and replaced with fresh.

Factor IX antigen was assayed in each sample by a quantitative ELISA assay and the results plotted as shown in Figure 4. It will be seen that the cells at 50% confluence or greater, secrete in the order of 100 $ng.ml^{-1}$ factor IX antigen per day (a level equal to about 2% of that found in normal serum).

The unusually high value on day 1 could be due to carry-over in the inoculum sample and the 200 $ng.ml^{-1}$ value found on day 6 represents the accumulated factor IX of 2 days secretion.

The following claims define some important aspects of the invention, but do not purport to include every conceivable aspect for which protection might be sought in subsequent continuing and foreign patent applications, and should not be construed as detracting from the generality of the inventive concepts hereinbefore described.

CLAIMS

1. Factor IX protein produced by recombinant DNA technology.

2. Human factor IX protein produced by recombinant DNA technology.

3. A process of preparing an artificial factor IX protein, which comprises preparing a recombinant expression vector, by linking a factor IX DNA sequence to a promotor sequence effective to express the DNA in a eukaryotic cell and incorporating these DNA sequences in a vector, and introducing the expression vector, in vitro, into eukaryotic cells having post-translational modifying means effective to modify the biologically inactive product of the expression of the DNA into biologically active factor IX protein.

4. A process according to Claim 3 wherein the factor IX DNA sequence comprises substantially all the cDNA sequence complementary to at least the coding part of factor IX mRNA.

5. A process according to Claim 4 wherein the DNA sequence further comprises a non-coding sequence to the 5'-end of the coding sequence.

6. A process according to Claim 3, 4 or 5 wherein the expression vector contains a gene providing a selectable marker for eukaryotic cells transfected with the vector.

7. A process according to any one of Claims 3 to 6 wherein the eukaryotic cells into which the expression vector is introduced are mammalian cells.

8. A process according to Claim 7 wherein the mammalian cells are of the dog kidney cell line MDCK.

9. A process according to any one of Claims 3 to 8 wherein the vector is introduced by transfection.

10. A process according to any one of Claims 3 to 9 wherein the factor IX DNA is human factor IX DNA.

11. A process according to any one of Claims 3 to 10 which further comprises recovering the biologically active factor IX protein from the eukaryotic cells and purifying it by affinity chromatography.

12. A process according to Claim 11 wherein in the affinity chromatography the factor IX protein is adsorbed on a supported antibody and desorbed by a high molar urea and high salt eluant.

13. A recombinant expression vector for use in a process defined in Claim 3, comprising a factor IX DNA sequence linked to a promotor sequence effective to express the DNA in a eukaryotic cell, incorporated in a vector.

ABSTRACT

FACTOR IX PROTEIN

The blood clotting protein, factor IX, is synthesised in the body in liver cells, where it undergoes three distinct types of post-translational modification before it is secreted into the bloodstream as a 415 amino acid long protein. It is therefore a difficult protein to produce by recombinant DNA technology. Nevertheless, such a result has been achieved by the present invention in which typically factor IX cDNA in a plasmid is linearised and inserted into an expression vector having a promotor sequence of SV40 early gene, an SV40 polyadenylation sequence, the TK/NEO selectable marker and an ampicillin resistance gene. Mammalian cells such as from a dog kidney or rat liver are transfected by the calcium phosphate precipitation method. Surprisingly high levels of factor IX, useful as a plasma-free preparation for treatment of patients suffering from Christmas Disease (haemophilia B), are obtainable.

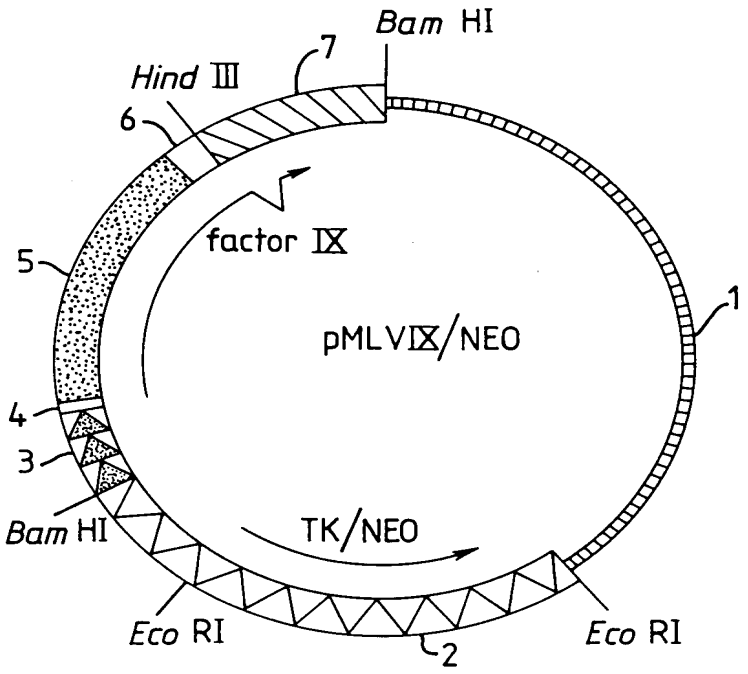
Bam HI
7
Hind III
6
factor IX
5
pMLVIX/NEO
1
4
3
Bam HI
TK/NEO
Eco RI
2
Eco RI

Fig. 1

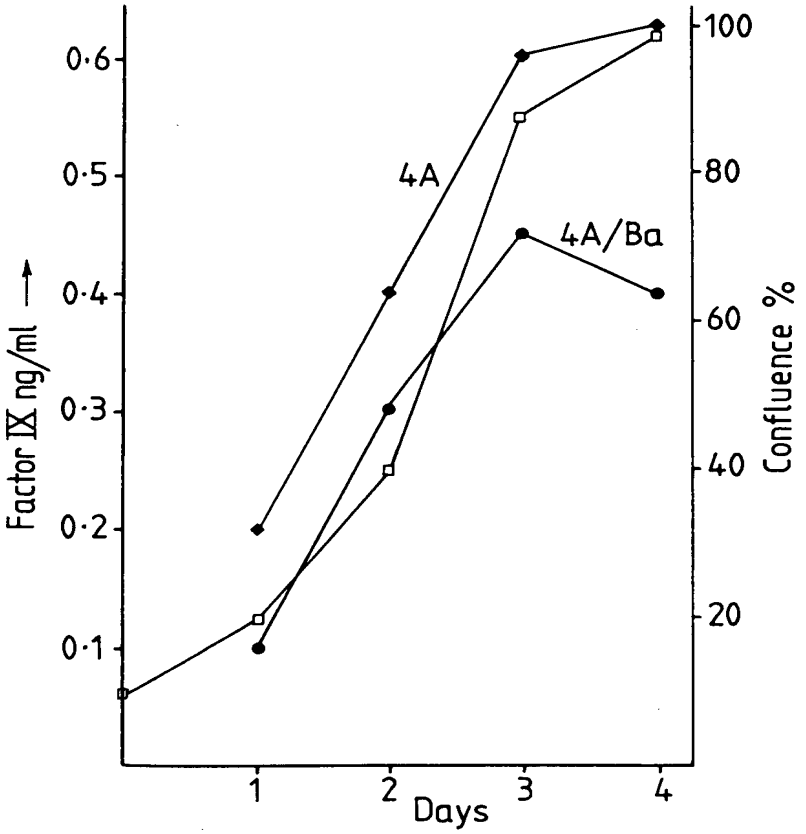
0·6
0·5
0·4
0·3
0·2
0·1
Factor IX ng/ml
4A
4A/Ba
100
80
60
40
20
Confluence %
1
2
3
4
Days

Fig. 2

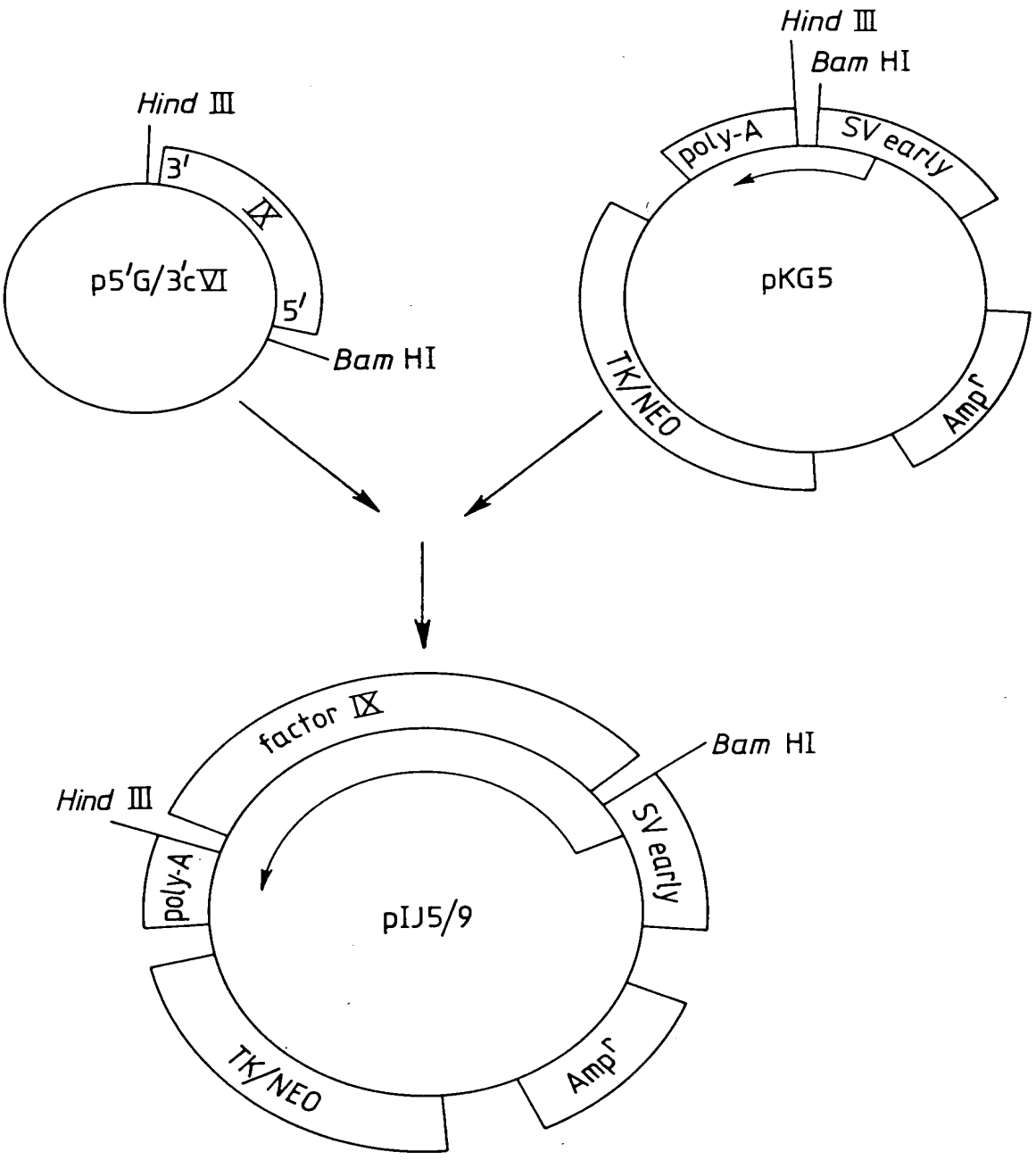
Hind III
Bam HI
poly-A
SV early
pKG5
TK/NEO
Ampr
Hind III
3'
IX
5'
p5'G/3'cVI
Bam HI
factor IX
Bam HI
Hind III
SV early
poly-A
pIJ5/9
TK/NEO
Ampr

Fig. 3

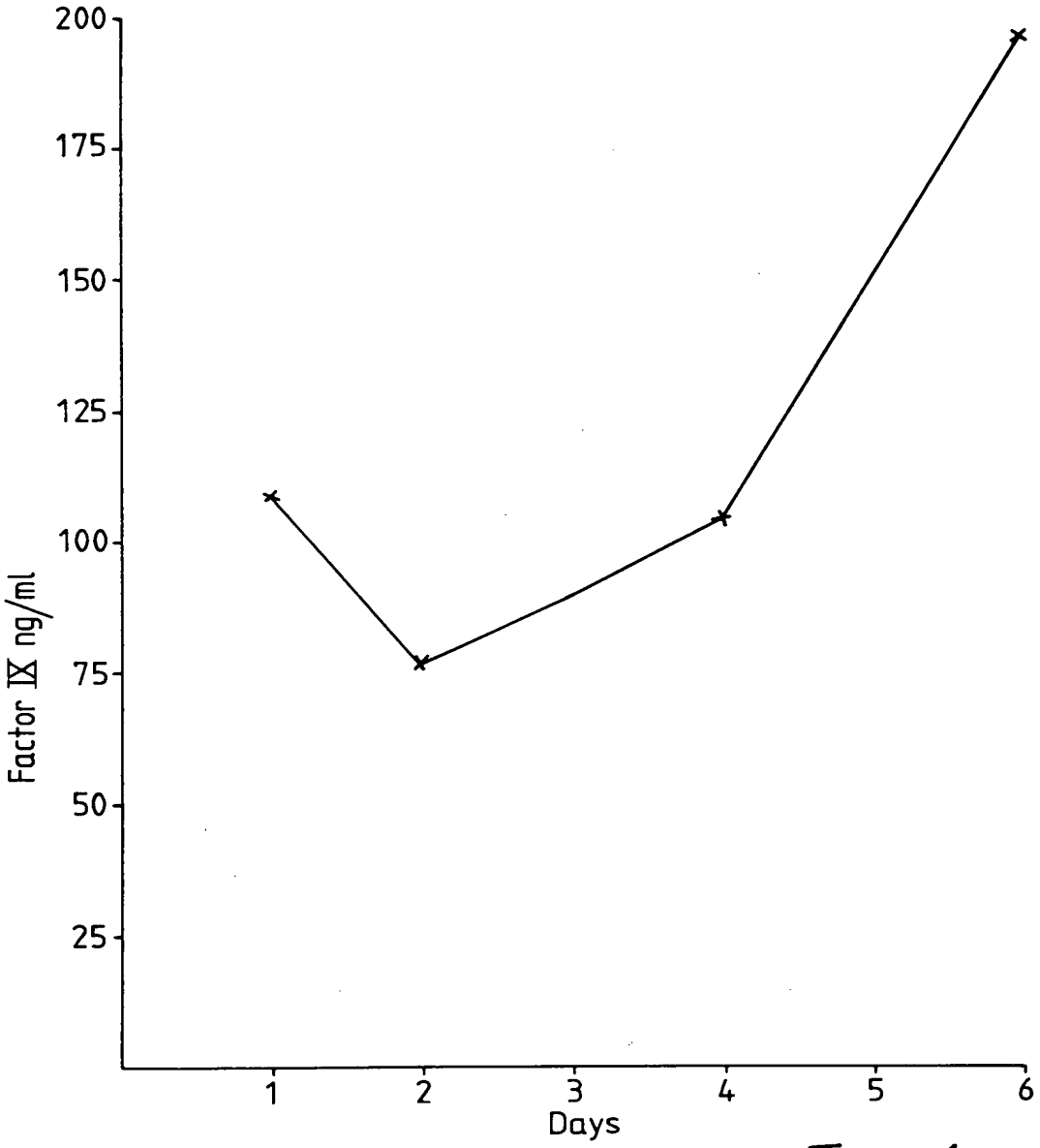

Fig. 4

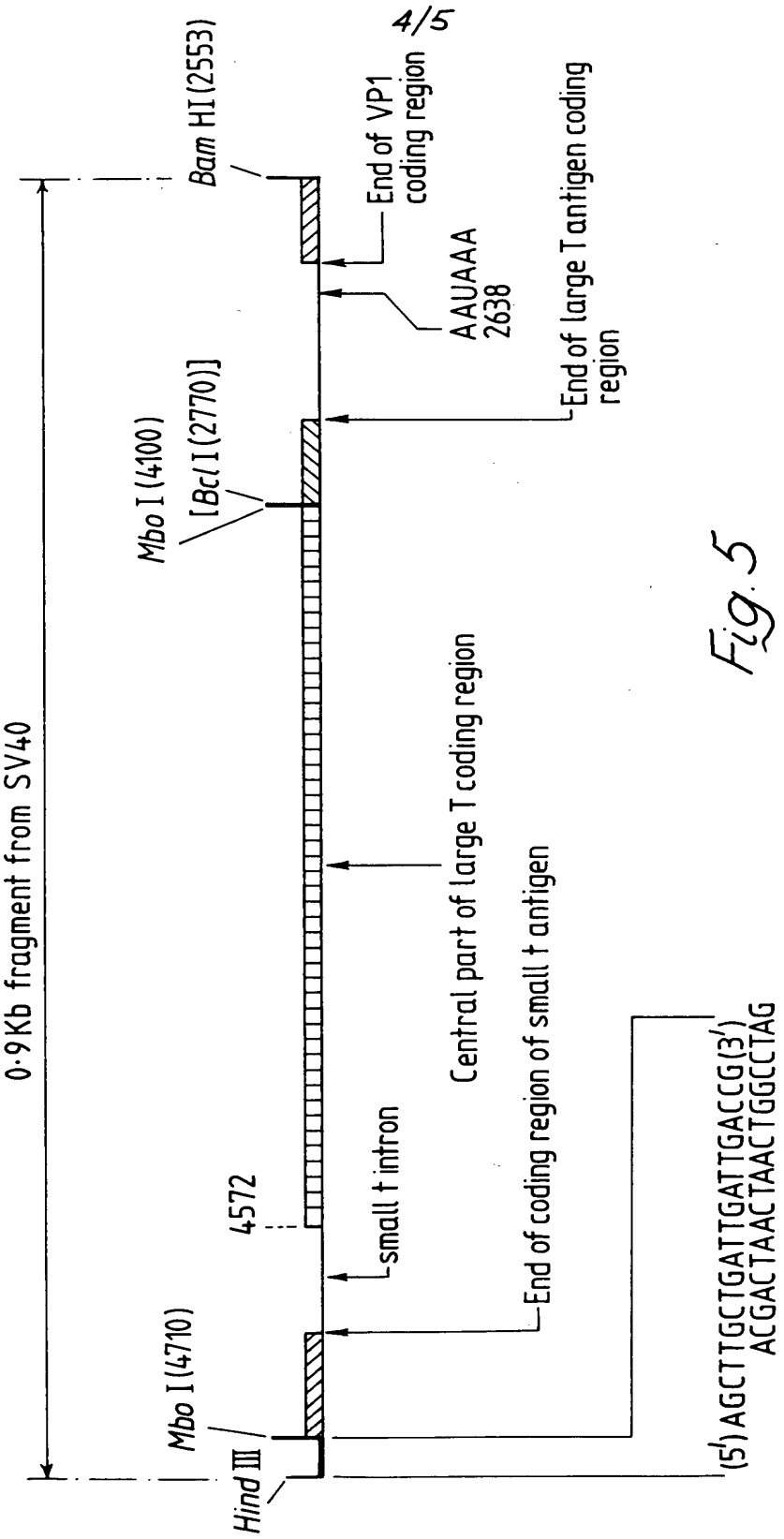
0·9 Kb fragment from SV40
Bam HI (2553)
End of VP1 coding region
AAUAAA 2638
End of large T antigen coding region
Mbo I (4100)
[Bcl I (2770)]
Central part of large T coding region
4572
small t intron
End of coding region of small t antigen
Mbo I (4710)
Hind III
(5') AGCTTGCTGATTGATTGACCG (3')
ACGACTAACTAACTGGCCTAG

Fig. 5

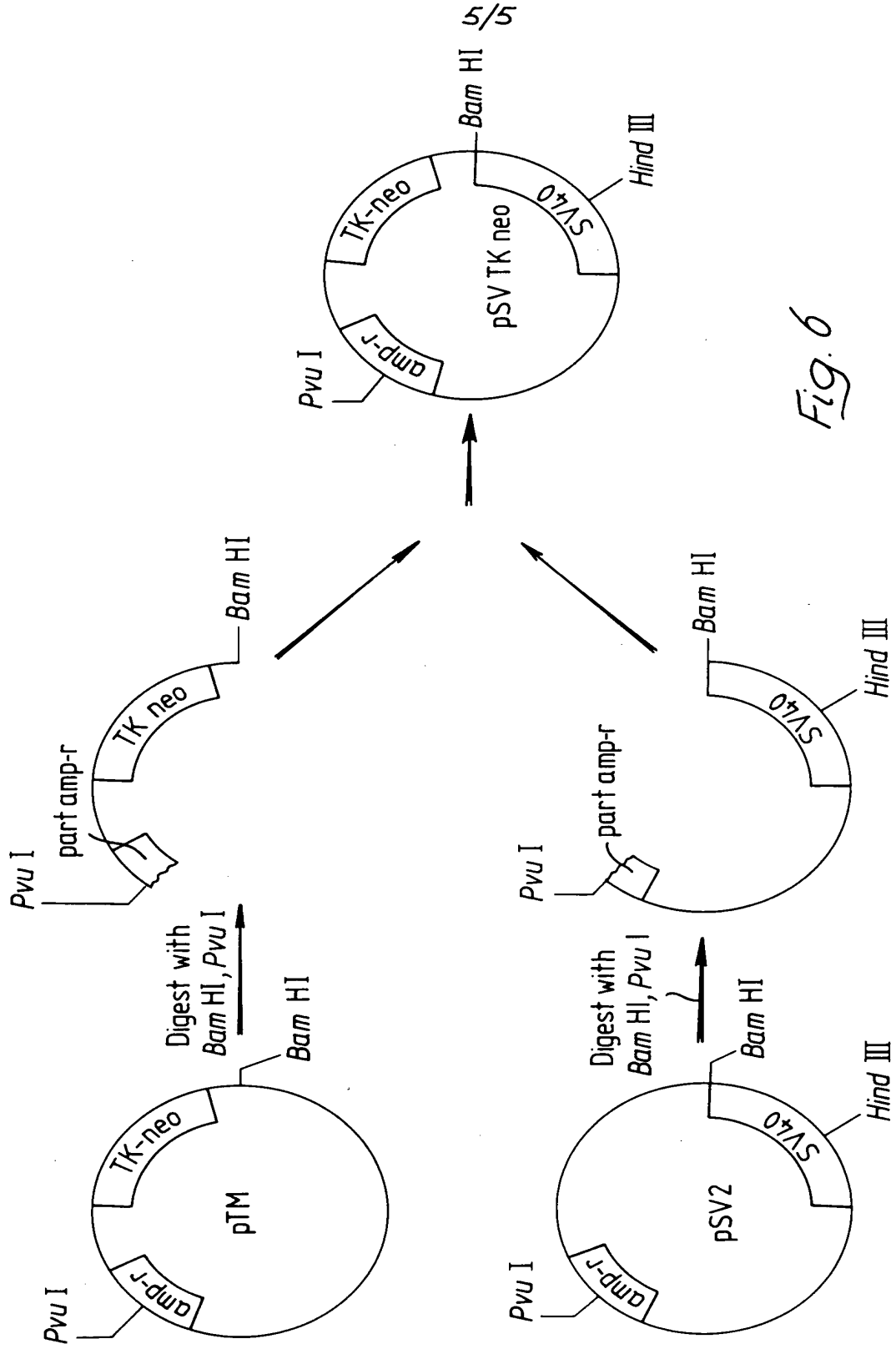
pTM
TK-neo
amp-r
Pvu I
Bam HI
Digest with
Bam HI, Pvu I
part amp-r
TK neo
pSV2
SV40
Hind III
pSV TK neo

Fig. 6